Report

Current Biology

Eight Millennia of Matrilineal Genetic Continuity in the South Caucasus

Highlights

- We analyzed 52 full mitochondrial genomes from ancient humans in the South Caucasus
- The results show a high level of maternal genetic continuity in this region
- Cultural shifts across eight millennia have not changed the maternal gene pool

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In Brief

Margaryan et al. analyze whole mitochondrial genomes of 206 modern and 52 ancient individuals that represent various cultural groups from the South Caucasus spanning eight millennia. The results clearly indicate genetic continuity of human maternal gene pool since Neolithic times despite well documented cultural shifts in the South Caucasus.

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Report

Eight Millennia of Matrilineal Genetic Continuity in the South Caucasus

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SUMMARY

The South Caucasus, situated between the Black and Caspian Seas, geographically links Europe with the Near East and has served as a crossroad for human migrations for many millennia [1–7]. Despite a vast archaeological record showing distinct cultural turnovers, the demographic events that shaped the human populations of this region is not known [8, 9]. To shed light on the maternal genetic history of the region, we analyzed the complete mitochondrial genomes of 52 ancient skeletons from present-day Armenia and Artsakh spanning 7,800 years and combined this dataset with 206 mitochondrial genomes of modern Armenians. We also included previously published data of seven neighboring populations (n = 482). Coalescence-based analyses suggest that the population size in this region rapidly increased after the Last Glacial Maximum ca. 18 kya. We find that the lowest genetic distance in this dataset is between modern Armenians and the ancient individuals, as also reflected in both network analyses and discriminant analysis of principal components. We used approximate Bayesian computation to test five different demographic scenarios explaining the formation of the modern Armenian gene pool. Despite well documented cultural shifts in the South Caucasus across this time period, our results strongly favor a genetic continuity model in the maternal gene pool. This has implications for interpreting prehistoric migration dynamics and cultural shifts in this part of the world.

RESULTS AND DISCUSSION

In this study, we present 206 mitochondrial genome sequences from three sub-populations of modern Armenians and 44 (plus eight previously published) mtDNA genomes from ancient individuals excavated in Armenia and Artsakh (Figure 1; Table S1). The calibrated radiocarbon dates of the ancient samples ranged between 300 and 7,811 years BP, with the majority being Bronze Age individuals, 3,000 to 4,000 years old (Table S1).

Shotgun sequencing data from all 44 ancient DNA extracts showed increased deamination damage rates at both 5′ and 3′ ends of sequencing reads compared to the revised Cambridge reference sequence (rCRS) reference mitochondrial sequence. The C→T transition rates at the first position of sequenced DNA fragments were between 8.9%–43.7%, indicating that the profiled DNA molecules were of ancient origin (Table S1). The estimated levels of DNA contamination were <8%, with an average of 1.3% across the entire ancient dataset (Table S1). Three pairs among the 44 ancient individuals had pairwise identical mitochondrial genome sequences (Table S2). Combined with archaeological data suggesting a close relationship (the same site and grave locations), these identical mtDNA sequences indicated a maternal relationship, and we therefore excluded data from one individual of each pair in most downstream analyses. Summary statistics and genetic diversity values for all groups are shown in Table S3. Negative Tajima's D values, observed for all four groups, could suggest a recent increase in population size [10].

The major mtDNA haplogroup frequencies in the four groups (three modern and one ancient) are presented in Figure 2, and qualitatively it is clear that the modern Armenian groups and the ancient group display obvious similarities. The three Neolithic samples (arm7, arm9, and arm39; ca. 7,800 years BP) in our dataset have mitochondrial haplogroups H and I, which have

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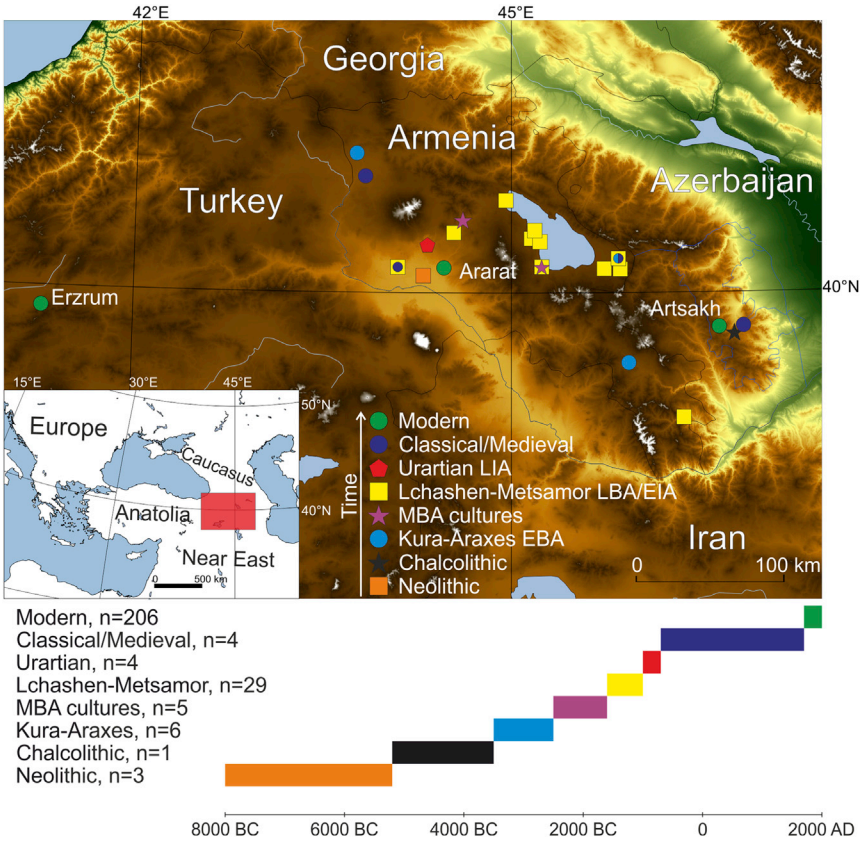

Figure 1. Study Area

Maps of the Near East (insert) and Armenia with sampling and origin areas of ancient and modern individuals, respectively. The legend shows the number of samples and the approximate time period covered by each cultural group. EBA, early Bronze Age; MBA, middle Bronze Age; LBA, late Bronze Age; EIA, early Iron Age; LIA, late Iron Age. See also Table S1.

previously been associated with the Neolithic expansion of farming cultures from the Near East [11]. Interestingly, haplogroup I, which first seems to appear in Europe during the Late Neolithic (ca. 4,000 years BP) [11], is observed in a Neolithic individual (arm39) from the South Caucasus, dated to 7,800 years BP. This early presence could reflect the geographic proximity of the South Caucasus to the putative place of haplogroup I origin in Southwest Asia [12, 13].

A correspondence analysis based on extended haplogroup frequencies of the ancient group, modern Armenians, and comparative populations (Africa, Europe, Caucasus, Near East, Central Asia, and East Asia) (Table S4) is presented in Figure S1. The plot clearly shows the clustering of the ancient group together with the modern European, Armenian, and Caucasian populations. We observe none of the typical East Eurasian mtDNA lineages (A, C, D, F, G, and M) among the ancient individuals, and only one individual with haplogroup D is present in the modern Armenian maternal gene pool (Artsakh). As such, the archaeologically and historically attested migrations of Central Asian groups (e.g., Turks and Mongols) into the South Caucasus [14, 15] do not seem to have had a major contribution in the maternal gene pool of Armenians. Both geographic (mountainous area) and cultural (Indo-European-speaking Christians and Turkic-speaking Muslims) factors could have served as barriers for genetic contacts between Armenians and Muslim invaders in the 11th–14th centuries CE. The same pattern was observed using Y chromosome markers in geographically diverse Armenian groups [16, 17]. An absence of East Eurasian mtDNA lineages in Armenia and Georgia was previously shown by Schönberg et al. [18], whereas in neighboring Turkic-speaking groups (Azeri and Turks), haplogroups A, C, D, F, G, and M7 are indeed present [18, 19], perhaps brought in by the Oghuz and Mongol migrations ca. 1,000 years ago.

We constructed a multidimensional scaling (MDS) plot based on the F_{ST} genetic distance matrix to visualize the genetic differentiation between our sample groups and seven other populations from the South Caucasus and the Near East for which complete mtDNA genome sequences data were available (Figure 3). The F_{ST} values show that the ancient individuals are genetically closest to the modern Armenian group from Erzrum and to modern Georgians (Table S5), and on the MDS plot they also cluster together with the three modern Armenian groups from Erzrum, Artsakh, and Ararat. The genetic distances between the ancient group and most of the included modern populations (F_{ST} values ranging from 0 to 0.0145) were not significantly different, indicating possible close genetic ties between ancient and most modern populations of the South Caucasus and northern parts of the Near East. We note that the sample sizes of several of the previously published comparative datasets were relatively small ($n < 30$), which could potentially cause a slight misrepresentation of the true genetic diversity of the source population. This is evident from the previously published smaller dataset of the Armenians [18], which does not capture the same diversity of mtDNA lineages (e.g., haplogroups R and I) as we document here in our larger dataset.

The genetic similarity between the ancient group and modern Armenians is also reflected in a TCS network of mtDNA haplotypes, a discriminant analysis of principal components, and the maximum-parsimony phylogenetic tree presented in Figure S2 and Data S1.

A Bayesian skyline plot (BSP) based on all modern and ancient mitochondrial genomes analyzed together revealed four putative demographic events (geometric mean of 4.4 with 95% highest posterior density intervals between 4 and 6 as obtained from the results of extended skyline plot analysis). The plot indicates a small but noticeable decrease in the effective female population size (N_e) around 25 kya during the Last Glacial Maximum (LGM), which is followed by a rapid (roughly 10-fold) population increase until around 10 kya (Figure 4A). The same result was

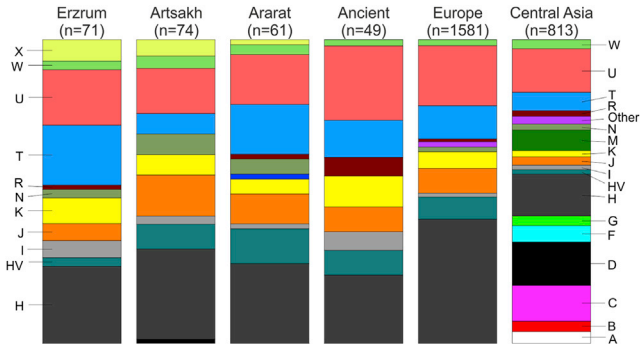

Figure 2. mtDNA Haplogroups

The figure shows the modern groups including three Armenian (Ararat, Artsakh, and Erzrum), Central Asian (Tajikistan, Kyrgyzstan, and Uzbekistan combined), and European (Italy-Tuscany, Poland, and Bulgaria combined) populations. "Other" denotes the combined frequencies of mtDNA haplogroups with less than 1% in Europe and Central Asia. Extended haplogroup frequencies and references for the European and Central Asian populations are presented in the Table S4. See also Data S1, Figure S1 as well as Tables S2 and S3.

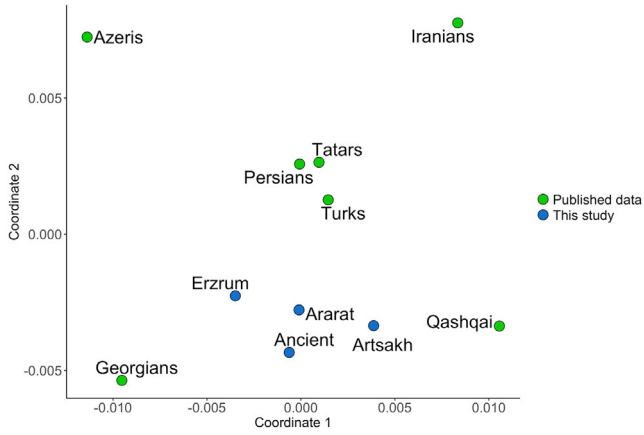

Figure 3. MDS Plot

The MDS analysis was based on F_{ST} genetic distances using whole mtDNA sequences of the ancient group (blue), modern Armenians (blue), and neighboring populations from the Near East and the South Caucasus (green). See also Table S5 and Figure S2.

observed when analyzing the data without partitioning the mtDNA sequences into mutation-dependent segments. This demographic trajectory is in accordance with previously published results based on data from European Mesolithic and Paleolithic individuals [20]. Interestingly, N_e appears to be declining around 5 kya, although the large confidence interval makes this conclusion tentative. This result was not observed in previous studies based on smaller samples size and modern data alone [18]. Interestingly, the timing of this putative decline coincides with the formation of complex societies during the Bronze Age in the region [21]. This could have increased susceptibility to diseases such as plague, which we know was present in both Central Asia and Europe during the early Bronze Age [22]. Another possibility is that the society formation of Bronze Age populations could have reduced the effective female population size without affecting the census population sizes. Factors like populations size fluctuations, increased selection, variation in family size, and changing population sub-structuring can all affect the estimates of effective population size [23]. However, it has previously been noted that recent population declines on BSP plots should be interpreted with caution as it may be an artifact of population structure [24].

Furthermore, we used approximate Bayesian computation (ABC) analyses to test five possible demographic model scenarios (Figure 4B), simulating 1,000,000 datasets from each model. For the modern group, we used the combined (n = 206) modern Armenian population (see the STAR Methods for the details of ABC analysis and rationale behind the model choices). The cross-validation of the ABC model selection is summarized in Table S3, showing that we can easily distinguish between the genetic continuity scenario (model 1) and the rest. We used two statistical tests, marginal density p value and Tukey depth p value, to assess the fit of our five models to the observed data. All models show high values for both statistics, indicating a good fit for all of them to the observed data (Table S3).

Based on comparison of the marginal densities, the analysis favor model 1 (posterior probability of 89% and Bayes factor of 8.1), which assumes genetic continuity between the ancient group and the modern Armenians (Table S3). This result suggests that there were no major genetic shifts in the mtDNA gene pool in South Caucasus across the last 7,800 years.

Using genetic data of modern Armenians, Haber et al. suggested that the Armenian gene pool was formed as a result of admixture events happening ca. 4,500 years BP [25]. Our ancient DNA (aDNA) data suggest that at least the maternal gene pool in the South Caucasus has been very stable and was largely formed before these events. A scenario of genetic continuity is supported by two previous studies that included low-coverage genomic data from a few ancient individuals from the South Caucasus: Allentoft et al. observed genetic similarities between Bronze Age individuals (ca. 3,500 years BP) and modern Armenians [26], and Lazaridis et al. showed similarity between Chalcolithic (ca. 6,000 years BP) and Bronze Age (ca. 3,500 years BP) individuals excavated in Armenia [7]. Moreover, Jones et al. presented results implying that such continuity might extend even further back in time: it appears that Upper Paleolithic Caucasus hunter-gatherers and Mesolithic individuals from the South Caucasus (Georgia) are genetically close to modern Caucasian groups, albeit also displaying their own genetic component [3]. The two hunter-gatherer individuals from this study had variants of mtDNA haplogroups H and K, which have typically been associated with later Neolithic times.

Our results have implications for how the known cultural shifts in the South Caucasus are interpreted. It appears that during the last eight millennia, there were no major genetic turnovers in the female gene pool in the South Caucasus, despite multiple well-documented cultural changes in the region [27, 28]. This is in contrast to the dramatic shifts of mtDNA lineages occurring in Central Europe during the same time period, which suggests either a different mode of cultural change in the two regions or that the genetic turnovers simply occurred later in Europe compared to the South Caucasus. More data from earlier Mesolithic cultures in the South Caucasus are needed to clarify this. During the highly dynamic Bronze Age and Iron Age periods, with the formation of complex societies and the emergence of

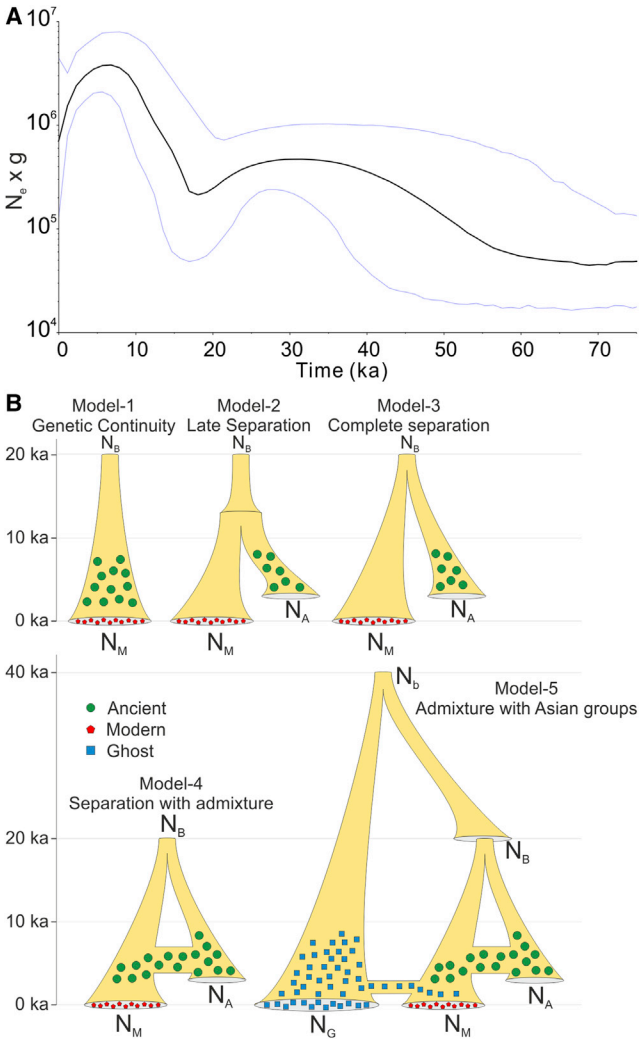

Figure 4. Population Demographics

(A) Bayesian skyline plot. Values on the y axis represent the effective female population size (N_e) × generation time (g).

(B) Schematic representation of the five demographic models used for coalescent ABC simulations. Population sizes of modern Armenians (N_M), ancient group (N_A), "ghost" Asian population (N_G), first bottleneck (20 kya; N_B), second bottleneck (40 kya; N_b) are shown.

For the details of ABC analysis and rationale behind the model choices, see STAR Methods. See also Table S3.

distinctive cultures such as Kura-Araxes, Trialeti-Vanadsor, Sevan-Artsakh, Karmir-Berd, Karmir-Vank, Lchashen-Metsamor, and Urartian, we cannot document any changes in the female gene pool. This supports a cultural diffusion model in the South Caucasus, unless the demographic changes were heavily male biased, as was most likely the case in Europe during the Bronze Age migrations [29, 30]. However, genome-wide data from the few Bronze Age individuals published so far from the South Caucasus also support a continuity scenario [26]. Another possibility is that any gene flow into the South Caucasus occurred from groups with a very similar genetic composition, facilitating only subtle genetic changes that are not detectable with the current datasets.

Due to the lack of available ancient and modern mtDNA genomes from other regions of the South Caucasus, we have here used Armenians as a representative group of the region. Considering the low and in many cases non-significant genetic differences that we observe between populations of the South Caucasus, one would expect to observe a somewhat similar pattern of matrilineal genetic continuity in other parts of this region, i.e., Georgia, Azerbaijan, and Armenian Highland (partially modern day Eastern Turkey and North-West Iran). Future studies should, however, prioritize to expand the sampling of both modern and ancient populations in the whole region to uncover the geographic and temporal extent of this genetic continuity signal.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, five tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.05.087.

AUTHOR CONTRIBUTIONS

M.E.A. initiated and led study. A.M., M.E.A., L.Y., and M.D. designed study. A.M., M.D., B.M., and G.D. produced the data. A.M., M.D., H.H., B.M., and R.H. analyzed the data. A.M., M.E.A., M.D., H.H., B.M., R.H., Z.K., L.Y., and E.W. interpreted results. A.M. and M.E.A. wrote the manuscript, with contributions from all authors. A.M., H.H., Z.K., P.A., R.B., A.B., V.M., G.S., A.P., H.S., R.M., G.D., and L.Y. excavated, curated, sampled, and/or described analyzed skeletons. All authors contributed to interpretation of data.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
44 ancient human bone and tooth samples	This paper	Table S1
206 modern Armenian saliva samples	This paper	Table S2
Deposited Data		
Raw fasta files of 52 ancient whole mitochondrial genomes	This paper	GenBank: MF362692–MF362743
Raw fasta files of 206 modern Armenian whole mitochondrial genomes	This paper	GenBank: MF362744–MF362949
Whole mitochondrial genomes of Azeris, Georgians, Iranians and Turks	[18]	N/A
Whole mitochondrial genomes of Persians and Qashqai	[19]	N/A
Whole mitochondrial genomes of Tatars	[31]	N/A
mtDNA haplogroup frequencies in Kenya	[32]	N/A
mtDNA haplogroup frequencies in Somalia	[33]	N/A
mtDNA haplogroup frequencies in Tajikistan, Kyrgyzstan and Uzbekistan	[34]	N/A
mtDNA haplogroup frequencies in Korea	[35]	N/A
mtDNA haplogroup frequencies in China_South Han	[36]	N/A
mtDNA haplogroup frequencies in Italy(Tuscany)	[37]	N/A
mtDNA haplogroup frequencies in Poland	[38]	N/A
mtDNA haplogroup frequencies in Bulgaria	[39]	N/A
mtDNA haplogroup frequencies in Jordan, Lebanon and Syria	[40]	N/A
mtDNA haplogroup frequencies in Adyghe, Chechnya and Kabardin	[4]	N/A
rCRS	*Homo sapiens* mitochondrion, complete genome	https://www.ncbi.nlm.nih.gov/nuccore/J01415.2
Software and Algorithms		
SeqScape 2.5 software	Applied Biosystems	http://tools.thermofisher.com/content/sfs/manuals/cms_041481.pdf
OxCal (Version 4.2)	Oxford Radiocarbon Accelerator Unit	https://c14.arch.ox.ac.uk/oxcal.html#program
CASAVA v.1.8.2	N/A	https://www.illumina.com/
AdapterRemoval 1.5.2	[41]	https://github.com/MikkelSchubert/adapterremoval
BWA 0.6.2	[42]	http://bio-bwa.sourceforge.net/bwa.shtml
Picard	http://picard.sourceforge.net	https://broadinstitute.github.io/picard/
Samtools	[43]	http://samtools.sourceforge.net/
genobox_bam2avgdepth1.py	GitHub	https://github.com/srcbs/GenoBox/blob/master/genobox_bam2avgdepth1.py
mapDamage 2.0	[44]	https://ginolhac.github.io/mapDamage/
contamMix	[45]	N/A
Haplogrep	[46]	http://haplogrep.uibk.ac.at/
Geneious v.9.1.3	N/A	http://www.geneious.com/
Arlequin	[47]	http://cmpg.unibe.ch/software/arlequin35/
R	R Development Core Team, 2016	https://www.r-project.org/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
POPART	http://popart.otago.ac.nz/index.shtml	http://popart.otago.ac.nz/index.shtml
mtPhyl v4.015	N/A	http://eltsov.org
PhyloTree Build 17	[48]	http://www.phylotree.org
BEAST v. 1.8.2	[49]	http://beast.bio.ed.ac.uk/
Python script for partitioning of mtDNA sequence	N/A	https://github.com/GrantHov/My_Python_codes
Tracer	[50]	http://tree.bio.ed.ac.uk/software/tracer/
jModelTest	[51]	https://github.com/ddarriba/jmodeltest2
Fastsimcoal2	[52]	http://cmpg.unibe.ch/software/fastsimcoal2/
Arlsumstat	[47]	http://cmpg.unibe.ch/software/arlequin35/
ABCtoolbox 2.0 (Beta)	[53]	https://bitbucket.org/phaentu/abctoolbox-public/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Morten E. Allentoft (meallentoft@snm.ku.dk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Modern samples

Saliva samples were collected from a total of 206 unrelated self-identified ethnic Armenians, whose ancestors (at grandparental level) originated from Artsakh (n = 74, individuals sampled in Stepanakert), Ararat (n = 61, individuals sampled in villages of the Ararat Valley), and Erzrum – modern Turkey (n = 71, individuals sampled in Yerevan). All subjects provided written informed consent for the collection of samples and subsequent analysis. Sampling was conducted by the scientific staff following an approval from the ethical board (IRB-00004079) at the Institute of Molecular Biology, NAS, Armenia.

Ancient samples

We sampled 44 ancient human skeletons from Armenia and Artsakh according to established aDNA guidelines [54] and sampling permissions were obtained from respective state institutions. A total of 19 archaeological sites are represented, covering large parts of Armenia as well as Artsakh (Figure 1), and estimated to be between 300–7800 years old based on contextual dating of artifacts. This time span is accompanied by at least seven well-defined cultural transitions: Neolithic, Chalcolithic, Kura-Araxes, Trialeti-Vanadzor 2, Lchashen-Metsamor, Urartian and Armenian Classical/Medieval (Figure 1).

Archaeological Sites

Aknashen

The site of Aknashen-Khatunarkh is located in the Ararat Plain, in the basin of the Sev dzhur (Metsamor), at an altitude of 838 m, in the province *(marz)* of Armavir (6 km south of Echmiadzine, on the northeast periphery of the village of Aknashen). The site is an artificial hill *(blur),* circular in plan, 100 m in diameter (a surface area of about 0.8 hectares), with a flat top rising about 3.5 m above the plain. The excavations of the site have been conducted since 2004 by the Armenian-French joint expedition.

The site belongs to the Late Neolithic "Aratashen-Shulaveri-Shomutepe" culture and dates back to the first half of the VI millennium BC. Human remains occur both from a cultural layer of the synchronous deliberately committed burial (Tr.6, UF 11, F.15) and of household waste (unburied remains of a newborn, Tr.4, UF 7a), and intrusive burial of the end of Early Bronze - the beginning of the Middle Bronze Age (second half of the III millennium BC; Tr.7, UF 5, F2).

Akunk

The cemetery is located in the area neighboring the eastern part of the village Akunq, Gegharquniq region. E. Khanzadyan carried out excavations here in 1970. Barrows were excavated, which contained one and more tombs with stone-cist and pit constructions. Tombs are related to the 9-8th centuries BC.

Artsvakar

The necropolis is located at the northern edge of the Artsvaqar region of the city Gavar. The Iron Age (11 – 9 centuries BCE) remains are found in the necropolis occupying about 0.1 squared kilometers. The tombs are earth and stone tumuli, 0.3-0.5 m high and having been built at ground level. Systematic archaeological excavations at the site have been conducted since 1992, and over 17 tombs dating from the Late Bronze Age (18 - 16 centuries BC) to Iron Age (12/11 – 9/8 centuries BC) period have been excavated there.

Azatan

The site is situated about 2 km south-west from the village Azatan (Shirak region) and takes an area of 1,5 km^2. It consists of a fortress, a settlement and 3 cemeteries, spread around them. Since 2011 till today it is studied by the Local-Lore Museum of Shirak and the archaeological expedition of the university Halle. The cemetery is dated to the Early Iron Age-Hellenistic periods. Pit, cist, stone-cist, catacomb, rock-cut tombs have been excavated. Pit burials are individual burials: The deceased are on left and right sides with the face to the east. Catacomb and rock-cut ones are sequential burials of some persons with traces of decarnation and human sacrifice.

Dari Glukh

The necropolis is located close to the city Gavar and is distributed on northern side of the Gavar – Noratus highway. The Late Bronze/Early Iron Age remains are found in the necropolis occupying about 0.2 km squared. Systematic archaeological excavations at the site have been conducted since 2003, and over 12 tombs dating to the Late Bronze/Early Iron Age (15 – 11 centuries BC) period have been excavated.

Godedzor

The settlement Nerkin Godedzor is situated 1.5 km west of the village Angeghakot, within the Vorotan river gorge, on the left bank of the river. Systematic excavations of the site have been realized during 2004-2014 by an Armenian-French team. The lower level of the site deals with the final stage of the Chalcolithic - transition to the Early Bronze Age (Kura-Araxes culture) and is dated to ca. 3600-3400 BC. The results of excavations demonstrate that it was a provisional settlement to be visited during summer season: in winter the population moved back to the lowlands, very probably situated on the southern shores of the Lake Urmia (Iran). The burials are within the settlement, in the pits made in clay-plastered floors of wooden-roof yards.

Kanagegh

The necropolis is located not far from Yeranos village, on southern shore of Lake Sevan. Distributed on both sides of the Sevan – Vardenis Highway. The Late Bronze Age – Iron Age remains are found in the south-western part of the necropolis, in a cemetery occupying about 0.3 km squared. Systematic archaeological excavations at the site have been conducted since 1982, and over 15 tombs dating from the Late Bronze Age (18 - 16 centuries BC) to Iron Age (12/11 – 9/8 centuries BC) period have been excavated in 1999-2002.

Kaps

The Early Bronze Age cemetery is situated in the northern part of the village Kaps (Shirak region), on the south-eastern slope of the eastern height of the homonymous reservoir. During the constructive works of the reservoir, the slope of ca. 60 m width was cut in order to open a new road and both 2 sides of the cutting destroyed stone-cist tombs became visible. The excavated tomb is 2,1 × 2,1 × 1,00 m in size. In the eastern wall of the tomb there was an entry. Three burials have been made: skeletons of two of them were collected in the north-eastern corner; the third one was on the left-side, along the southern wall. A finger-ring of bronze, as well as two black-burnished vessels were found in the tomb.

Karkar

The fortress-city Karkar is situated between the cities Stepanakert and Shushi, on the cape of the right bank of the river Karkar. The area of the site is 40-45 ha. The settlement was protected by a cyclopean masonry from the south, and by a three-stepped ground barrier and water-ditch from the north. The excavations attested that the city was settled since the 8-7th centuries BC till the 14th century AD. At the beginning of the 18th century a small fort-post was founded on the hill of the citadel, in the ruins of the round tower of which a secondary burial was found, carried out after the destroying of the tower, probably in the 18th century AD. A bone from this burial was given for expertise.

Karashamb

The Karashamb necropolis is situated 1.6 km south of village Karashamb, in Kotayk Province, on one of the terraces of the right bank of Hrazdan Gorge (lat. – 40 23 49.3 N, long. – 44 35 28.8 E). Today the preserved segment of the necropolis occupied an area of about 3.5 hectares and is only a fraction of the previous larger cemetery. Systematic archaeological excavations at the site have been conducted since 1981 – 1984, 2009 – 2015, and over 1500 tombs dating from the Middle Bronze through Iron II Ages period (the last quarter of the III millennium BCE to the second quarter of the 1st millennium BC).

Burials NN 50, 462 and 750 were excavated during archaeological field works in 2010 and 2013. These are attributed to the middle (second) phase (20 – 18 centuries BC) of the Trialeti-Vanadzor culture, which is dated from the last quarter of the III millennium BC to the early part of the II millennium BC (23 – 16 centuries BC).

Karintak

This cave is located in the Shushi Region (Artsakh), in the midst of the lush Karintak Forest (which derives its name from a nearby village). The name 'Karintak' is apt as it means 'beneath the rock'. The Grid Reference for the location is N 39.74320° E 46.76611° and the elevation is approximately 1,400 m.

Karintak cave contains two separate cave passages (termed Cave 1 and Cave 2). Cave 1 is considerably longer, extending for approximately 42 m in north-east direction (inward from the entrance). The passage continues deeper, beyond this point, but is too narrow to pass through. Cave 1 actually narrows and widens (constriction and necking) several times along its length, producing a set of small 'sub-chambers'. Cave 2 extends in from the entrance in a westerly direction for only c. 10 m before turning 90° in a southerly direction for another c. 5 m.

Karintak cave is still fairly wet today. Stalactites, stalagmites and flowstone were observed in the rear of Cave 1.

Archaeological survey of the site has been conducted since 2014, a 2 m long x 1 m wide x 1.8 m deep test pit approximately 30 m from the cave mouth was excavated. The human tooth used for this study was excavated in 2015 in the Cave 1.

Metsamor

The cemetery of Metsamor is situated in the south-eastern part of the city Metsamor. In 2011 one destroyed tomb was excavated here. It is located not far from the highway Yerevan-Armavir, on a natural hill. Excavations demonstrate that an Early Iron Age tomb was on the natural hill, in which later - a new burial was done to be destroyed during the construction of the city.

Nerkin Getashen

The necropolis is located at Nerqin Getasehen village, and is distributed on both sides of the Yerevan – Sevan-Vardenis highway. The Middle Bronze Age to Iron Age remains are found in the southern part of the necropolis, in a cemetery occupying about 0.7 squared kilometers. Systematic archaeological excavations at the site have been conducted since 1905, and over 67 tombs dating from the Middle Bronze Age (22/21 – 17/16 centuries BC) through Iron Age (13/12 – 9/8 centuries BC) period have been excavated.

The Middle Bronze Age is represented by 7 tombs dated to the 19 – 22 centuries BC and belong to the Trialeti-Vanadzor and Sevan-Artsakh cultures (II-III phases of the Middle Bronze Age). The tombs are earth and stone tumuli, 0.5-1.5 m high and had been built at ground level.

The Late Bronze Age –Iron Age is represented by 58 tombs. These are dated to the 15/14 – 9/8 cc. BCE and belong to the Lchashen-Metsamor culture. The tombs are earth and stone tumuli, 0.3-1.5 m high, having been built at ground level and excavated in 1989-1991.

Nerqin Naver

Nerqin Naveri necropolis is situated near Parpi, Oshakan, Voskevaz villages in Aragatsotn Province around 1100 m above sea level and was discovered in 1978. On the east side of burial field linearly located 5 tumuli. Tumulus N 1 was excavated during archaeological field works between 2002-2003 and had multiple layers. In the top layer (80 cm) pieces of pottery and a horse iron bridle was found while in the second layer (80-120 cm) human hand and foot phalanges were discovered. In the third layer (120-200 cm) in the north-eastern part of the burial, human long bones, phalanges and teeth were excavated which didn't have anatomical positioning. According to the anthropologists, these bones belonged to two individuals. Pieces of jewelry, silverware and sacrificed animal bones were also discovered in this layer. In deeper layers (200-250 cm) scattered animal bones of many species, jewelry and pottery was present. At the bottom of this layer human teeth from both children and adults were found. This burial is attributed to the Iron Age.

Sevan

The excavation was conducted in 1956 by an expedition of the Historical Museum of the Academy of Sciences headed by H. Mnatsakanyan. On the dried territories of the lake Sevan (Tsamakaberd) more than 20 burials had been excavated. Tomb 19 is oriented to the north-east to south-west; the chamber is a stone cist with longer sides (two slabs), and shorter (one stone slab) ones from northern and southern parts. The tomb was covered by two stone slabs, one of which was demolished with waters of the lake. Inner part of the chamber was covered with limestone sediment solidified thickness of about 30 cm. Bones here were well preserved more than in the other graves. The skeleton was lying on the left side with bent arms and legs. The excavated material is dated to 12th cent BC.

Sotk

There are two main archaeological site in Sotk: Sotk 1 and Sotk 2, excavated durint 2011-2015 by an Armenian-German team. The site Sotk 1 is located in the southern part of the village Sotk, Gegharkunik Region, RA, and is a fortress-settlement with intrusive tombs within the settlement. The site comprises on the whole an area of ca. 1.5 ha. The fortress-settlement dates from the Achaemenide and Artasheside periods (ca. 500-1 BC), the intrusive tombs without inventory are surely Medieval (perhaps ca. 500-1500 BC). Systematic archaeological excavations at the site have been conducted during 2014 in various parts of the fortress-settlement. We deal with a road-guiding fortress in which burials have been made during Medieval period, when the fortress was abandoned.

The site Sotk 2 is located in northern part of the village Sotk, Gegharkunik Region, RA, and is a fortress-settlement with tombs within the settlement. The site comprises on the whole an area of ca. 2 ha. The settlement dates from the Early to Late Bronze Ages (ca. 3000-1200 BC), the fortress from the Middle and Late Bronze Ages (ca. 1600-1200 BC). The intramural pit-graves are contemporary with the settlement. Systematic archaeological excavations at the site have been conducted during 2011-2015 in various parts of the fortress-settlement guiding the road to the gold mines of Sotk and the road to Karvachar. The sample derives from the intramural child burial belonging to the Early Bronze Age.

METHOD DETAILS

Modern samples

Samples from the modern Armenian individuals (n = 206) were sequenced at the Genetics Laboratory of Institute of Biological Problems of the North, Russian Academy of Sciences, Magadan, Russia.

Genomic DNA was extracted using a standard phenol/chloroform procedure. The entire mtDNA was amplified in 11 overlapping PCR fragments, using a set of primers with matching annealing temperatures as described in detail by Torroni et al., 2001 [55]. After PCR, the fragments were purified using the DiatomTM DNA Clean-Up kit (Isogene Laboratory), and Cycle Sequencing was performed by application of BigDye Terminator v3.1 chemistry (Applied Biosystems), using set of 32 nested primers specifically designed for this protocol [55]. An ABI 3500xL sequencer was used for separation of the sequencing ladders. Complete mtDNA sequences were aligned and assembled using the software SeqScape 2.7 (Applied Biosystems). The obtained complete mtDNA sequences were compared to the revised Cambridge reference sequence (rCRS) [56] and assigned to haplogroups based on human mtDNA phylogeny presented in mtDNA tree Build 17 (18 February 2016) (http://www.phylotree.org).

A total of 482 whole mtDNA sequences from seven populations from the South Caucasus and the Near East were also used as a comparative dataset [18, 19, 31].

aDNA extraction

The ancient DNA (aDNA) work was conducted in dedicated aDNA clean-room facilities at Centre for GeoGenetics, Natural History Museum, University of Copenhagen according to strict aDNA standards [54, 57]. The vast majority of ancient samples (41 out of 44) were teeth, but one petrous bone and two postcranial bones were also used (Table S1). We targeted the endogenous DNA rich outer layer of the tooth roots for ancient DNA extraction [58].

All the samples were mechanically cleaned to minimize contamination from modern DNA by removing the surface area with diamond-dust-coated disk using a drill. For the teeth samples, only the outer cementum-rich part of the roots was used for DNA extraction by removing the crown and root dentine with a cutting disk and pointy drill-bit respectively [58]. For the few petrous bone samples, we targeted the otic capsule region which was previously shown to have the highest levels of endogenous DNA [59]. The drilled bone material (ranging from 200 to 400 mg) was briefly digested (predigestion step) by incubating in digestion buffer (4.65 mL 0.5 M EDTA, 50 μL recombinant Proteinase K, 50 μL 100x TE and 250 μL 10% N-Laurylsarcosyl) for 45 min at 40°C. After this predigestion step [58], the samples were centrifuged, and the supernatant was removed after which an identical digestion buffer was applied for a full 24 hr digestion at 40°C. After this step, the samples were centrifuged and the remaining pellets were stored for later re-extraction. The DNA was isolated from 2 mL digested solution using a silica-powder-based extraction method. The silica suspension was prepared by mixing 6g of SiO2 with 50 mL H2O. After 1 hr of sedimentation, 48 mL supernatant was transferred to a new 50 mL tube followed by another 5 hr sedimentation. Then the top 43 mL was removed and the silica was re-suspended and activated with 60 μL 37% HCL. To each of 2 mL digested sample, 20 mL of the binding buffer (19.54 mL QIAGEN buffer PB, 360 μL 5M sodium acetate, 100 μL 5M sodium chloride) and 100 μL silica suspension was added and adjusted to pH 4-5 with 37% HCl [26]. After a 1 hr incubation at room temperature the supernatant was removed after a brief centrifugation step at 2000 g for 2 min and the pelleted silica was re-suspended in 1 mL binding buffer and washed twice with 80% cold ethanol. The DNA was eluted from silica particles in 60 μL QIAGEN EB buffer. Extraction blanks were included with each round of extractions.

NGS library preparation and sequencing of ancient samples

DNA extracts (20 μl) were build into blunt-end libraries using Illumina-specific adapters and NEBNext DNA Sample Pre Master Mix Set 2 (E6070) kit according to manufacturer's instructions with some modification mentioned below:

Since ancient DNA molecules are already naturally fragmented, the nebulization step was skipped. The end-repair step was carried out in 25 μL reactions using 20 μL of DNA extract. This was incubated for 20 min at 12°C and 15 min at 37°C, and purified using PB buffer with QIAGEN MinElute spin columns, and eluted in 17 μl. Next, Illumina-specific adapters according to Meyer and Kircher 2010 [60] were ligated to the end-repaired DNA in 25 μL reactions. The reaction was incubated for 15 min at 20°C and purified with PB buffer on QIAGEN MinElute columns, before eluted in 20 μL EB Buffer. The adaptor fill-in reaction was performed in a final volume of 30 μL and incubated for 20 min at 65°C followed by 20 min at 80°C to inactivate the Bst enzyme. To assess the amount of DNA libraries in each sample and therefore the optimal number of PCR cycle for library amplification, qPCR was performed using SYBR green MIX (Roche) according to manufacturer's instructions and the same forward and reverse primers used for the following index PCR step. The DNA library (12 μl) was then amplified and indexed in a 50 μL PCR reaction, mixing with 25 μL 2X Kapa U+, 1 μL of each primer (10 μM, inPE forward primer + indexed reverse primer) and 11 μL H_2O. Thermocycling conditions were 45 s at 98°C, followed by number of cycles (based on qPCR values) of 15 s at 98°C, 30 s at 65°C and 30 s at 72°C, and a final 1 min elongation step at 72°C. The amplified library was purified with PB buffer on QIAGEN MinElute columns, before being eluted in 50 μL EB. Negative library controls, constructed on EB, were included, as well as libraries constructed on the negative extractions controls.

Sequencing of aDNA

The purified DNA libraries were quantified on an Agilent Bioanalyzer 2100. The library pools were sequenced (100 bp, single read) on Illumina HiSeq 2500 system at the Danish National High-throughput DNA Sequencing Centre. Basecalling and sequence sorting by sample-specific indexes were performed by the Sequencing Centre using CASAVA v.1.8.2.

We sequenced the 44 complete ancient mitochondrial genomes to an average depth of 11.7x to 159.7x (Table S1) which allowed us to apply strict quality filters for calling the consensus mtDNA sequences. After re-mapping the sequencing reads of an additional eight published samples [26] to the rCRS, we obtained an average mtDNA depth of 7.4x to 268x for these (Table S1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Carbon dating

To confirm archaeological ages of the samples, 10 skeletons from different sites were radiocarbon-dated at the Department of Physics & Astronomy, Aarhus University (Table S1). Mean calibrated ages (years BP) with 95.4% low and high probability intervals of n = 10 ancient samples were obtained by OxCal (Version 4.2; Oxford Radiocarbon Accelerator Unit).

Basic bioinformatics

Adaptor sequences and stretches of Ns at both ends were trimmed from all of the ancient DNA reads using AdapterRemoval 1.5.2 [41], keeping only sequences with a minimum length of 30 bp. The trimmed sequences were mapped against the human mitochondrial genome reference (rCRS) using BWA 0.6.2 aligner [42] with the seed disabled allowing higher sensitivity [61]. The aligned sequences were filtered for mapping quality 30 and sorted using Picard (http://picard.sourceforge.net) and samtools [43]. Duplicate sequences at library level were removed by Picard MarkDuplicates (http://picard.sourceforge.net). Read depth was determined by a python script genobox_bam2avgdepth1.py available at Github. We also extracted sequencing reads mapping to rCRS from eight previously published ancient Armenian samples [26] and analyzed them together with our 44 new samples. The consensus mtDNA sequence for each sample was called using an in-house perl script that considers only bases with a base quality score of 20 and sites with a sequencing depth of at least 5X. At each position a base was called only if it was observed in at least 70% of the reads covering that site [62].

Authentication of aDNA

DNA damage parameters, namely the C → T transition rates that are typical for aDNA [63], were estimated using mapDamage 2.0 [44] with default settings. The levels of human DNA contamination in the ancient samples were assessed with contamMix [45]. This software estimates for each ancient sample how well the mtDNA reads map to the consensus mtDNA sequence of the same sample compared to a collection of 311 worldwide mitochondrial genomes. The mtDNA consensus was called with the same parameters (and custom perl script) as described above.

Haplogroups

Mitochondrial haplogroups of the ancient individuals and the modern Armenian individuals were assigned using haplogrep [46]. The complete consensus mtDNA sequences of the ancient individuals were aligned with the modern Armenian data and comparative datasets using Geneious v.9.1.3. Genetic diversity indexes, neutrality tests and F_{ST} genetic distances were calculated with the Arlequin software package [47]. The prcomp and CA (FactoMineR package) functions in the R statistical software were used to conduct MDS and correspondence analysis, respectively. We used the "DAPC" R package to conduct the discriminant analysis of principal components (DAPC) [64]. A phylogenetic network analysis of the ancient individuals and the modern Armenians was conducted with POPART (http://popart.otago.ac.nz) using the TCS algorithm.

Phylogenetic tree construction

The maximum-parsimony phylogenetic tree of ancient and modern Armenian complete mtDNA sequences was constructed using the mtPhyl v4.015 software (http://eltsov.org). Since this software uses earlier version of PhyloTree (Build 11) as its reference phylogeny, the tree was modified manually to take into account the updated mtDNA phylogeny presented in PhyloTree Build 17 [48]. Numbers along links refer to substitutions scored relative to the rCRS [56]. Transversions are further specified; ins and del denote insertions and deletions of nucleotides, respectively; back mutations are underlined; symbol < denotes parallel mutation; heteroplasmies are italicized and labeled using the IUPAC code; underlined positions with question marks correspond to back mutations at haplogroup-specific sites whose status is unknown due to unresolved bases in certain parts of the ancient DNA sequences. For phylogeny reconstruction, the nucleotide position 16519 as well as positions showing point indels and/or transversions located between nps 16180-16193, 303-315, 522-524, 573-576 were not used. Armenian samples are labeled as in Table S2. Established haplogroup labels are shown in black; blue are redefined and red are newly identified haplogroups in the present study.

Effective population size

To uncover the trajectory of the effective female population size (N_e) through time, we applied the Bayesian Skyline plot (BSP) method [65] implemented in BEAST v. 1.8.2 [49]. We constructed the BSP using both modern and ancient mitochondrial genomes in a combined dataset, using the ages of the latter as calibration points in the tree inference.

We partitioned the alignments into four datasets with a partition scheme adapted in accordance with a recently published molecular clock calibration for human mitochondrial DNA [66]: i) First and second nucleotides in codons of protein coding genes (PC1+PC2), ii) third nucleotides in codons of protein coding genes (PC3), iii) rRNAs+tRNAs, and iv) HVRI+HVRII. Indels were removed from the alignments to avoid potential biases due to possible misalignments and incorrect consensus calling around these regions. Partitioning was made by an in-house Python script, available through our Github account https://github.com/GrantHov/My_Python_codes. Beauti [49] was used for generating the XML input files for BEAST v. 1.8.2. When running BEAST, we used unlinked strict clock rates and substitution models for the four partitions but linked the generated trees. The following substitution models were used based on results of jModelTest v.2.1.10 [51] with the Bayesian Information Criterion: HVRI+HVRII – TrN+I+G; PC1+PC2 – TrN+I; PC3 – TrN+G; rRNAs+tRNAs – HKY+I. Every partition was assigned an independent mutation rate prior according to Rieux et al., 2014 [66]. We ran the MCMC chains for 10E8 states, sampling every 10E4 states, and designating the first 10E6 states as burn-in. The BEAST runs were performed using the CIPRES open-access server for phylogenetics studies [67]. We checked the output data for convergence to a stationary distribution and sufficient effective sample size estimates using Tracer v. 1.5 [50].

Demographic model testing

We simulated data and tested five possible demographic scenarios for their fit to our observed dataset using Approximate Bayesian Computation (ABC). Fastsimcoal2 [52] was used to create 10E6 simulations for each model scenario. The summary statistics of simulated and observed data were calculated with Arlsumstat [47]. We used seven different summary statistics for the ABC analyses: number of polymorphic sites (S), mean number of pairwise differences (π) and Tajima's D (D) calculated for the ancient individuals and modern Armenians separately, and the F_{ST} genetic distance between the modern and ancient groups. We combined the three modern Armenian sub-populations into one and for all models, a generation time of 25 years and a mutation rate of $5.5x10^{-7}$/site/generation was used [66]. The ancient individuals were simulated with the time points corresponding to their calibrated ^{14}C-age or archaeological age estimates.

The first four models assume a bottleneck c. 800 generations ago at the end of the last glacial maximum (LGM), in accordance with the general consensus and our results from the skyline analysis in BEAST. In Model 1 (*Genetic Continuity*), this bottlenecked maternal population gives rise to the ancient South Caucasus population which is directly ancestral to the modern Armenian population of size N_M. In Model 2 (*Late Separation*), the initial small group gives rise to two different populations representing ancestors to our sampled modern and ancient groups. This divergence occurs 300-799 generations ago, sometime after the LGM and before the sampling of our oldest individual. In Model 3 (*Complete Separation*), the initial maternal population splits into two groups right after the LGM forming the modern and ancient populations, respectively. Model 4 (*Separation with Admixture*) is similar to Model 3, but with an admixture event between the two lineages 200 generations ago involving 1% to 90% of the gene pool. The time for admixture event was chosen around the time of population density increase and formation of the Armenian language [21, 68]. Model 5 (*Admixture with Asian groups*) starts with a bottleneck of a non-European maternal population around 2000 generations ago (50 kya) which eventually gives rise to a population that is ancestral to our modern and ancient sampled groups as well as an Asian lineage (ghost population). The demographic relationship of the ancient group and the modern Armenian group is here similar to Model 4, but we simulate an additional admixture event (1% to 90%) 50 generations ago (around the time of Arab, Mongol and Turkic invasions) from the Asian ghost population into the modern Armenian maternal gene pool.

For estimating the posterior densities of all parameter values, we used ABCtoolbox 2.0 (Beta) (https://bitbucket.org/phaentu/abctoolbox-public/) [53]. The same software package was used to assess the fit between the simulated data from each of our five different models to the observed data. This was done by calculating marginal density and Tukey depth p values with 1000 retained simulations. The former estimates the fraction of the retained simulations whose marginal density is equal or smaller than that of the observed summary statistics, while the latter statistic indicates the fraction of simulations whose Tukey depth is lower or equal to the Tukey depth of the observed data. We used the cv4postpr function implemented in the ABC R package to cross-validate the ABC model selection with 100 pseudo-observed datasets.

DATA AND SOFTWARE AVAILABILITY

Raw fasta files of 52 ancient and 206 modern Armenian full mitochondrial genomes have been deposited in GenBank under accession numbers MF362692–MF362949.

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Supplemental Information

Eight Millennia of Matrilineal Genetic Continuity in the South Caucasus

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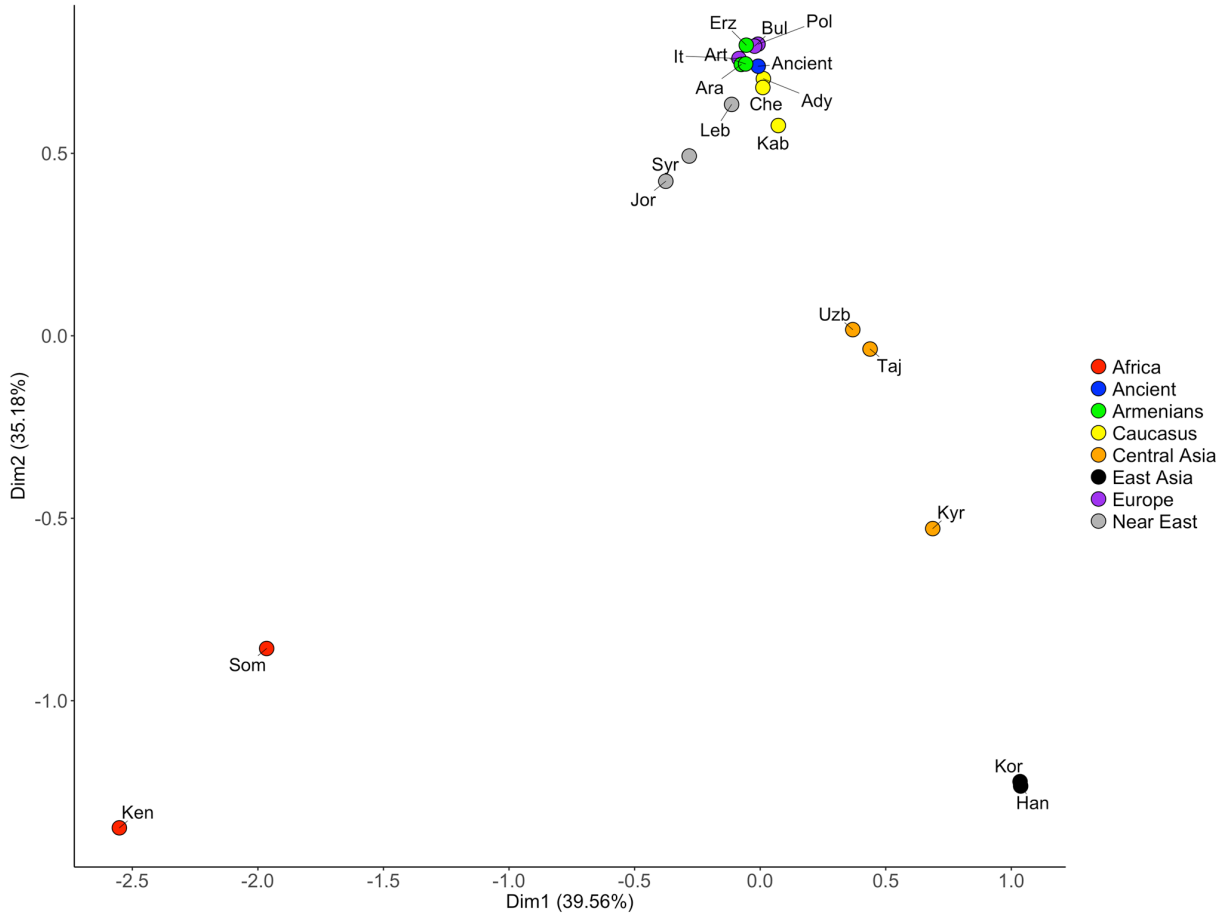

Figure S1. Correspondence analysis based on mtDNA extended haplogroup frequencies,

Related to Figures 2 and 3

Ken – Kenia[S1]; Som – Somalia[S2]; Taj – Tajikistan[S3]; Kyr – Kyrgyzstan[S3]; Uzb – Uzbekistan[S3]; Kor – Korea[S4]; Han – China_South Han[S5]; It – Italy(Tuscany) [S6]; Pol – Poland[S7]; Bul – Bulgaria[S8]; Jor – Jordan[S9]; Leb – Lebanon[S9]; Syr – Syria[S9]; Ady – Adyghe[S10]; Che – Chechnya[S10]; Kab – Kabardin[S10]; Ara – Ararat (Armenian: This study); Erz – Erzrum (Armenian: This study); Art – Artsakh (Armenian: This study); Ancient: This study. Haplogroup frequencies are provided in Table S4.

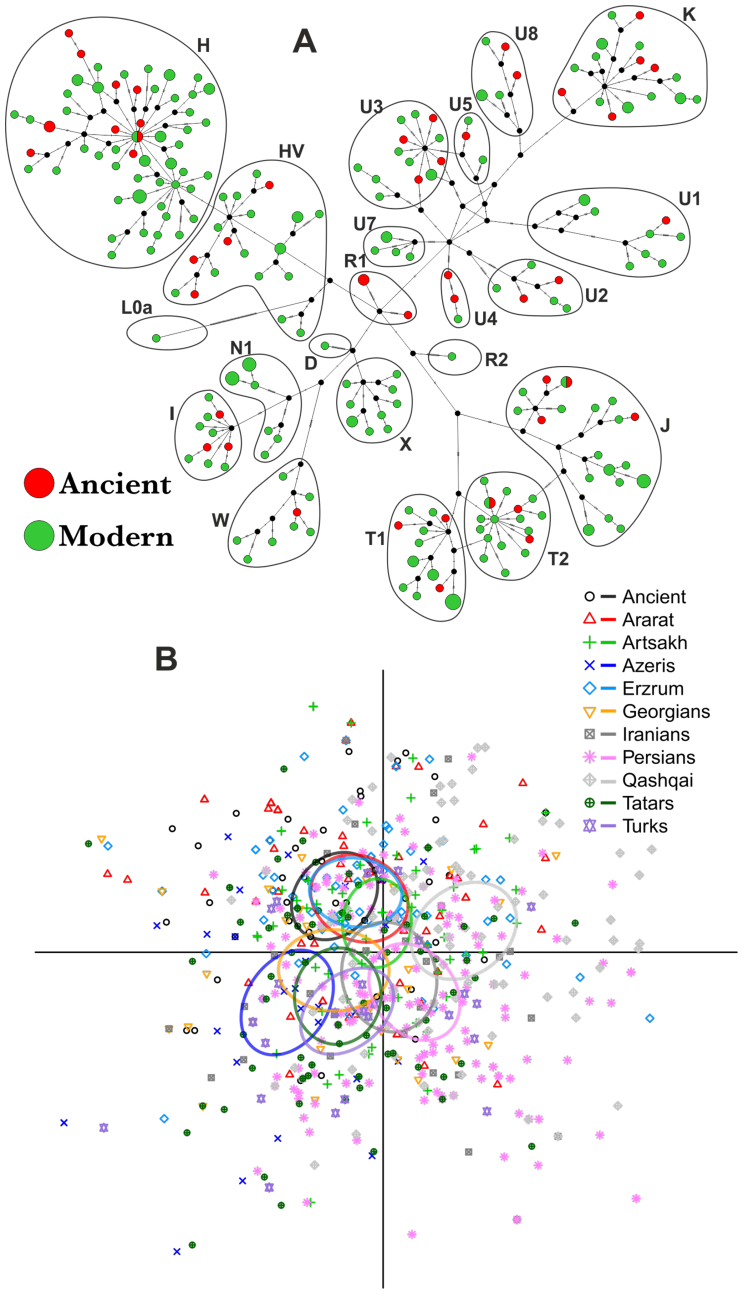

Figure S2. mtDNA network analysis and discriminant analysis of principal components, Related to Figures 2 and 3

(A) TCS network analysis based on whole mtDNA sequences of the ancient individuals (red) and modern Armenians (green). The major mtDNA haplogroups are indicated. The three Armenian sub-populations are pooled into one group for this analysis. (B) Discriminant analysis of principal components (DAPC) plot based on 737 complete mitochondrial genome sequences. Colored dots represent individuals while the ellipses are based on inertia statistics (cellipse=0.5). The two axes represent 88.5% of total variation. The ancient group (black ellipse) shares much of its variation with the modern Armenian groups (red, light-blue and green ellipses) followed by the Georgian cluster (orange).

(A)

Group	n	s	Gene diversity	k	π	Tajima's D
Ancient	49	339	1±4e-3	30.8±13.7	0.0019	-2.17*
Ararat	61	454	1±3e-3	35.0±15.5	0.0021	-2.28*
Artsakh	74	515	1±2e-3	33.7±14.9	0.002	-2.38*
Erzrum	71	437	1±2e-3	33.7±14.9	0.002	-2.2*

(B)

	Model-1	Model-2	Model-3	Model-4	Model-5
Model-1	95	3	0	2	0
Model-2	8	45	27	14	6
Model-3	2	27	44	11	16
Model-4	11	20	30	26	13
Model-5	1	9	20	11	59

(C)

Model	Posterior probability	Marginal density p-value	Tukey depth p-value	Tukey depth	Bayes factor
Model-1	0.89	0.998	0.998	0.4	8.066
Model-2	0.018	1	0.998	0.378	0.019
Model-3	0.016	0.998	0.999	0.393	0.016
Model-4	0.016	1	0.998	0.36	0.017
Model-5	0.06	0.998	0.999	0.37	0.063

Table S3. Genetic diversity indexes, ABC model choice and model choice cross-validation, Related to Figures 2 and 4

(A) Genetic diversity indexes in the ancient and 3 modern Armenian groups. n – number of individuals in each group; s – number of polymorphic sites; k – mean number of differences between all pairs of sequences; π – nucleotide diversity; * $p<0.001$ (B) ABC model choice cross-validation based on 100 samples from each model. The numbers show the frequency (%) of simulations of each model (mentioned in columns) that were chosen by abc while using pseudo-observed summary statistics from a randomly chosen simulation from the five models mentioned in the rows. (B) ABC Model choice results obtained by abctoolbox.

Table S1, Table S2, Table S4, Table S5 and Data S1 have been uploaded as separate excel files.

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